APPLICATIONS

Effective Sample Preparation and LC-MS/MS Analysis of Unconjugated Bile Acids from Human Serum

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Sensitive and effective methods for the extraction and analysis of bile acids from human serum were developed using an Impact™ protein precipitation plate and Kinetex® 2.6µm Polar C18 HPLC/UHPLC column.

Introduction

Bile acids are 24 carbon steroids formed in the liver from cholesterol and are essential to solubilize and promote absorption of dietary lipids and vitamins. The most abundant primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA), while abundant secondary bile acids include deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) which are formed by deconjugation and dehydroxylation in the colon. Numerous analytical methods have emerged to determine bile acid concentration from plasma and serum in recent years.[1-3] However, the major challenge of baseline separation between isobaric species remains, as LC-MS/MS alone doesn't allow for identification between them. In this application, we developed an LC-MS/MS method for the quantitation of 8 unconjugated bile acids utilizing a high efficiency Kinetex 2.6µm Polar C18 core-shell column to ensure baseline resolution of each key isobaric group of bile acids. For sample clean-up and extraction, a quick and easy method was developed using an Impact protein precipitation 96-well plate.

Materials and Methods

Reagents and Chemicals

Double charcoal stripped human serum (DC Mass Spect Gold, MSG 4000) was purchased from Golden West Biological (Temecula, CA). HPLC-grade acetonitrile and methanol were purchased from Honeywell (Morris Plains, NJ). All analytes and internal standards along with other chemicals were obtained from the Sigma-Aldrich Company (St. Louis, MO). Purified water was obtained using a Sartorius arium® comfort II, courtesy of Sartorious Corporation (Bohemia, NY).

Experimental Conditions

The LC-MS/MS method uses a Kinetex 2.6µm Polar C18 core-shell 100 x 2.1 mm column with a 2 mM ammonium acetate (pH 6.9) and methanol/acetonitrile (50/50) mobile phase. The LC gradient resulted in a total run time of 9.5 minutes. Detection was carried out on a SCIEX Triple Quad™ 4500, equipped with ESI source operating under negative polarity. An Impact protein precipitation 96-well plate was used to extract the analytes from serum sample. To achieve reproducible and accurate results, a set of 8 stable isotope labelled bile acids were used as internal standards (**Table 1**). A double charcoal stripped serum sample was employed for extraction purposes to minimize potential bias due to the presence of any endogenous bile acids.

Sample Preparation

Product Name:	Impact™ Protein Precipitation 2 mL Plate
Part No.:	CE0-7565
Size:	96-Well Plate
Add:	400µL methanol into the wells of Impact plate
Load:	100 µL of Serum sample (spiked with internal standards and analytes at 200ng/mL) directly into the organic solvent in each well of the plate.
Vortex:	2 minutes at maximum speed (use a sealing mat to prevent cross well contamination in the plate)
Wait:	Allow 5 minutes for completion of protein precipitation
Filter:	**Centrifuge:** Place the Impact plate on top of a collection plate and centrifuge at 500 g for 5 minutes or until filtrate is collected. **Vacuum:** Place the Impact plate onto a suitable 96-well sample manifold or robot. Ensure that a 96-well collection plate is positioned inside the manifold or under the Impact plate. Vacuum at 5 inch Hg for up to 5 minutes or until filtrate is collected. **Positive Pressure:** Place the Impact plate on top of a collection plate and apply 2-5 psi using a positive pressure manifold.
Dilute & inject:	Dispense 300µL of mobile phase A (or water) into the collection plate, vortex for 30 secs at a high speed and inject on LC-MS/MS.

LC-MS/MS Conditions

Column: Kinetex 2.6µm Polar C18
Dimension: 100 x 2.1 mm
Part No.: 00B-4759-AN
Recommended Guard: AJ0-9530
Mobile Phase: A: 2 mM Ammonium Acetate (pH 6.9)
B: Methanol/Acetonitrile (50/50)

Gradient

Time (min)	B (%)
0	45
9	70
9.5	70
9.51	45
12.0	45

Flow Rate: 400µL/min
Temperature: 50 °C
Injection Volume: 5 uL
System: Agilent® 1260
Detection: MS/MS ESI- (SCIEX Triple Quad™ 4500)
Analytes: See Table 1

Results

Figure 1. Chemical structure of the 8 bile acids from the analyte panel

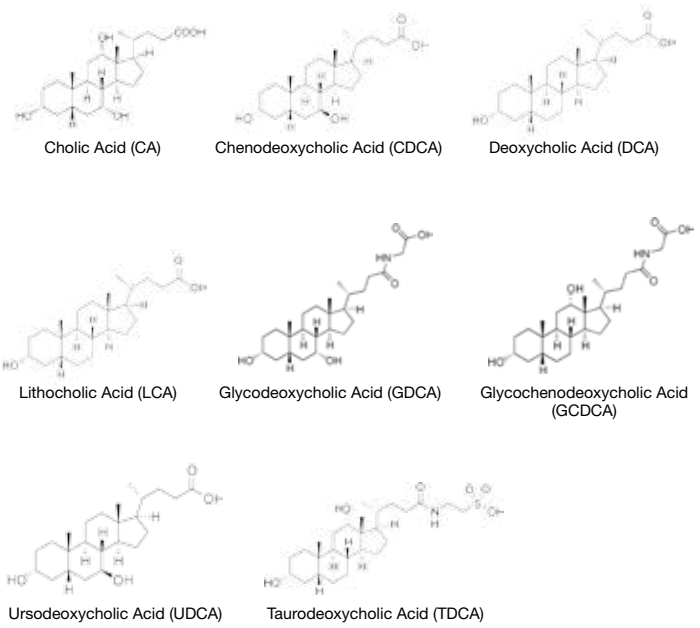

Figure 2. Representative TIC chromatogram of unconjugated bile acids extracted from human serum sample, utilizing an Impact PPT Plate

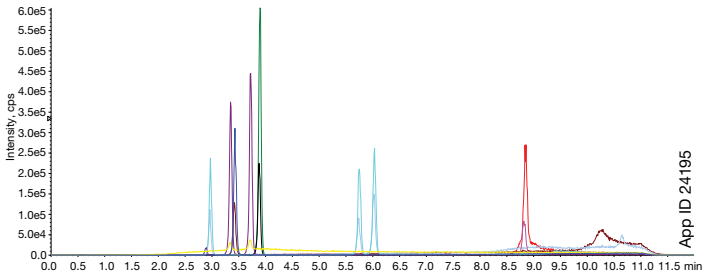

Figure 3. Representative XIC chromatogram displaying baseline separation of the three isomeric bile acids, UDCA, CDCA, and DCA

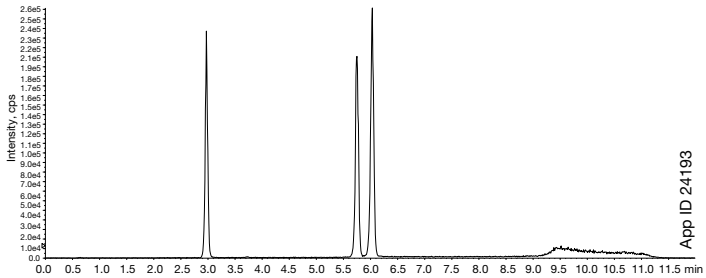

Figure 4. Representative XIC chromatogram displaying baseline separation two isomeric bile acids, GCDCA and GDCA

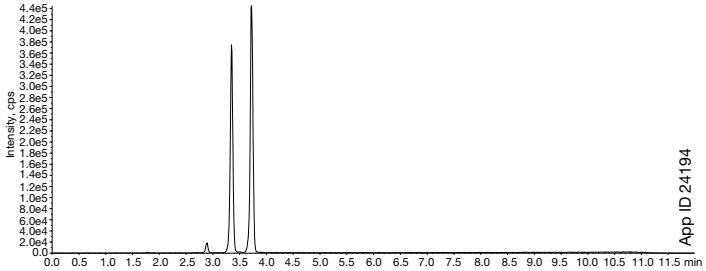

Table 1. Retention and Recovery of the Bile Acids

Analyte & IS name	RT(min)	% Recovery	% CV (N=5)
UDCA	2.97	91%	1.1
UDCA-D4	2.96		
GCDCA	3.35	90%	3.7
GCDCA-D4	3.34		
CA	3.43	88%	4.8
CA-D4	3.42		
GDCA	3.72	90%	4.4
GDCA-D4	3.71		
TDCA	3.90	94%	3.5
TDCA-D4	3.88		
CDCA	5.75	90%	4.5
CDCA-D4	5.74		
DCA	6.03	88%	4.6
DCA-D4	6.02		
LCA	8.90	90%	6.9
LCA-D4	8.80		

Discussion

The bile acid panel studied in this application contains several isomeric analytes such as UDCA, CDCA, DCA, GCDCA and GDCA that behave identically due to their structural similarity and can't be distinguished by mass spectrometry alone. Therefore, it is imperative that optimized chromatographic conditions be established to identify and confirm these isomers. As a result, a shallow gradient from 45 % to 70 % was employed (for mobile phase B) over 9 minutes to aid in the separation and sharpening of the isomeric peaks of interest (figure 3 and 4). Within the LC-MS method development a 2 mM ammonium acetate (pH 6.9) was found to be more effective over 0.1 % formic acid as a modifier, resulting in

a higher MS response for all analytes of interest. Additionally, the use of the Kinetex 2.6 µm Polar C18 LC column provided the necessary resolution between the bile acids of interest by means of a dual non-polar and polar selectivity and high column efficiency.

The serum concentration range expected for the analytes of interest in this assay are quite high (≥ 1500 ng/mL) and a 1:1 dilution of the post extracted samples provided more than enough signal for MS analysis. The dilution step prevents the peak distortion or early elution in the solvent front, circumventing the need for time intensive evaporation and reconstitution of the samples. **Table 1** shows high recovery (88 % to 94 %) for all BA analytes tested. The Coefficient of variation (CV) value for 5 replicate extraction came down less than 7 %, demonstrating good reproducibility of the assay. The oleophobic membrane (organic solvent leak resistant for at least 30 minutes) loaded Impact plate, enables gradual protein precipitation and optimum recovery in one single step, bypassing the need for centrifugation.

Conclusion

We conclude herewith, a combined simple and efficient sample extraction and LC-MS/MS method for quantitation of 8 unconjugated bile acids. The Impact sample preparation solution produces fast turnaround that demands virtually no method development and can easily be transcribed to automation. For analysis, the Kinetex Polar C18 column provided the necessary increased retention and resolution between the bile acids of interest through a combination of core-shell particle performance advantages and a highly useful dual non-polar/polar selectivity.

References

1. A. Zhu, W. Lu, E. Epure; Rapid Quantitation of 15 Major Bile Acids in Human Serum by UPLC-ESI-MS/MS; MEDPACE Bioanalytical Laboratories
2. M. Schere, C. Gnewuch, G. Schmitz, G. Liebisch, Rapid quantitation of bile acids and their conjugates in serum by liquid chromatography-tandem mass spectrometry; Journal of Chromatography B, 877 (2009) 3920-3925.
3. L. Luo, S. Schomaker, C. Houle, J. Aubrecht; Evaluation of Serum Bile Acid Profiles as Biomarkers of Liver Injury in Rodents; Toxicological Sciences.

Kinetex® Ordering Information

2.6 µm Minibore Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	**30 x 2.1**	**50 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
Polar C18	00A-4759-AN	00B-4759-AN	00D-4759-AN	00F-4759-AN	AJ0-9532 for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	**30 x 3.0**	**50 x 3.0**	**100 x 3.0**	**150 x 3.0**	**3/pk**
Polar C18	—	00B-4759-Y0	00D-4759-Y0	00F-4759-Y0	AJ0-9531 for 3.0 mm ID

2.6 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	**30 x 4.6**	**50 x 4.6**	**100 x 4.6**	**150 x 4.6**	**3/pk**
Polar C18	—	00B-4759-E0	00D-4759-E0	00F-4759-E0	AJ0-9530 for 4.6 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000.

Impact™ Ordering Information

Part No.	Description	Unit
Impact Precipitation Products		
CE0-7565	Impact Protein Precipitation, Square Well, Filter Plate, 2 mL	2/pk
CE0-7566	Impact Protein Precipitation, Square Well, Long Drip, Filter Plate, 2 mL	2/pk
Impact Starter Kit for Protein Precipitation		
CE0-8201	Impact Protein Precipitation Plate (CE0-7565) (2 ea) Collection Plate 2 mL (2 ea) Sealing Mat, Santoprene™ (AH0-8199) (2 ea)	ea

guarantee

If Phenomenex products in this technical note do not provide at least an equivalent separation as compared to other products of the same phase and dimensions, return the product with comparative data within 45 days for a FULL REFUND.

APPLICATIONS

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